PHOTODYNAMIC INACTIVATION OF BACTERIOPHAGE AND ITS INHIBITION

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The photosensitizing action of dyes on biological systems is commonly designated as "photodynamic action." The subject has been well summarized in a monograph by Blum (1941). Earlier studies on photodynamic inactivation of bacteriophages were made by Clifton (1931), and by Perdrau and Todd (1933). Burnet (1933) discovered that the relative sensitivities of phage strains to photodynamic inactivation could be correlated with serological groupings, and hence was of taxonomic significance. Quantitative studies of the kinetics of photodynamic inactivation of bacteriophages by Welsh and Adams (1954) confirmed Burnet's conclusions. The experiments undertaken in the present study were designed to find the conditions necessary for the photodynamic inactivation of bacteriophages. A mechanism for the inactivation or its inhibition in the T-series of coliphages will be described.

MATERIALS AND METHODS

Escherichia coli strain B and the T-series of coliphages (T1, T2, T3, T4, T5, T6, and T7) were used in this study. The techniques described by Adams (1950) were employed for all experiments. The saline buffer diluent contained NaCl, 0.58 per cent; $MgSO_4$, 0.025 per cent; and was buffered at pH 7.0 with sodium and potassium phosphate.

The dyes used were Merck's and Grübler's medicinal. They were dissolved in distilled water at a concentration of 0.01 M and stored as stock solutions. The light source was a 20-watt Matsuda fluorescent day-light lamp (40 cm long) with reflector. The lamp was fixed at a distance of 15 cm above the mixtures to be irradiated. All experiments were performed at night using a dim bulb which caused no detectable inactivation of photosensitized phage. Before and after the process the samples were held in a completely dark chamber.

The phage suspensions used were diluted in saline suspensions of the dyes to 5×10^7 particles per ml. Irradiation was carried out in a glass dish in which the liquid level did not exceed 1.5 mm. The dishes were shielded with black paper and tape during irradiation to eliminate reflected and indirect light. During the irradiation, samples were taken at various intervals and immediately diluted in buffered saline, then assayed for infective phage particles by the agar layer method. A light control of phage illuminated in saline without dye was performed. For a dark control, samples of the phage-dye solutions were held in the dark chamber under the same environmental conditions as in inactivation experiments.

RESULTS

Preliminary experiments. Preliminary experiments confirmed several of the results of Burnet (1933), and Welsh and Adams (1954). These were: (1) Photodynamic inactivation of phage is an exponential function of time, with an initial, somewhat variable lag, usually corresponding to that of a 2 or 3 hit curve (Welsh and Adams, 1954). The velocity constant k for photodynamic inactivation can be calculated from the slope of the exponential part of the curve. (2) The photodynamic inactivation rate increases as the pH increases and the salinity decreases (Yamamoto, 1956). It may be inferred that the photodynamic inactivation of phage requires the ionic bonding of the dye with the dye-receptor site of the phage particle, and that the more efficient the combination, the higher the inactivation rate. (3) The velocity constant of photodynamic inactivation under anaerobic conditions is negligibly small as compared to that of aerobic conditions. (4) The relative sensitivity of phage strains to photodynamic inactivation is correlated with their serological classification.

Photodynamic activity of various dyes. Twenty-five dyes belonging to seven classes were tested. The measurements of photodynamic inactiva-

TABLE 1

Photodynamic activity of various dyes on coliphage T5

Dye*	pH†	Velocity Constant (min^{-1})
I. Azo dyes		
Methyl orange	5.3	0.028
	7.4	0.000
Bismark brown	7.0	0.002
	7.4	0.039
II. Quinonimide dyes		
Methylene blue	7.0	0.574
	7.4	0.765
	8.0	1.027
Toluidine blue	7.4	0.397
Methylene green	7.4	0.024
Thionine	7.4	0.102
Azur I	7.4	0.704
Azur II	7.4	0.362
Brilliant cresyl blue	7.0	0.179
	7.4	0.259
	8.0	0.429
Neutral red	7.0	0.035
	7.4	0.008
	8.0	0.073
Safranine	7.4	0.001
	8.0	0.095
III. Phenylmethane dyes		
Malachite green	8.0	0.041
Rosaniline	5.3	0.038
	7.0	0.020
Methyl violet	8.0	0.073
Crystal violet	8.0	0.06
Methyl green	7.4	0.006
Victoria blue	7.4	0.135
IV. Xanthine dyes		
Pyronine G	7.4	0.050
Eosine	5.3	0.029
V. Acridine dyes		
Acridine orange	7.4	0.301
	8.0	0.365
Acriflavine	7.4	0.246
	8.0	0.279
Rivanol	7.4	0.101
IV. Cyanine dyes	7.0	0.056
	7.0	0.000
VII. Dye related substance		
20-Methyl cholanthrane	7.4	0.018
	8.0	0.088
No dye	8.0	0.035

* Dye concentration: M/60,000. Phage concentration: 5×10^7 particles/ml.

† pH Are medium conditions tested.

tion (PI) were carried out using M/60,000 dyes in buffered saline. The coliphage T5 was used. The results of these experiments are summarized in table 1. Among the various dyes tested, the quinonimides, such as the thiazine and oxazine dyes, and the acridine dyes were found to be most active. These dyes have properties in common and analogous structures. The dyes showing only slight activity ("inactive" dyes) may only be capable of exciting oxygen present, and the excited oxygen in turn inactivates the phage through an oxidative mechanism. When catalase was used as an inactivator of excited oxygen the action of inactive dyes was inhibited, but not the action of active dyes.

PI of different phages by various dyes. Seven strains of the coliphage T-series belonging to four different serological groups were tested for their sensitivity to PI by various dyes. The results are shown in table 2. The following order of photodynamic activity of the active dyes can be constructed.

T1: methylene blue > toluidine blue > acridine orange, acriflavine, brilliant cresyl blue.

T3 and T7: acridine orange > methylene blue > toluidine blue > acriflavine > brilliant cresyl blue.

TABLE 2

Photodynamic activity of various dyes on the T-series of coliphates

Dye	Velocity Constant (min^{-1})						
	Serological groups						
	Strain						
	T1	T2	T4	T6	T3	T7	T5
Methylene blue.	0.24	0.01	0.02	0.01	0.55	0.72	0.77
Toluidine blue..	0.12	0.00			0.36	0.57	0.40
Brilliant cresyl blue.....	0.07	0.00			0.18	0.33	0.26
Neutral red....	0.01	0.01	0.01		0.06		0.01
Crystal violet..		0.00			0.04		0.06
Methyl green...		0.00					0.01*
Pyronine.....		0.01			0.02		0.05
Acridine orange.	0.06	0.00	0.00	0.00	0.97	1.15	0.35
Acriflavine.....	0.04	0.01			0.29	0.46	0.28
Rivanol.....		0.01					0.10
No dye.....		0.02					

The buffered saline: pH 7.4.

Dye concentration: M/60,000. Phage concentration: 5×10^7 particles/ml.

* pH: 8.0.

T5: methylene blue > toluidine blue > acridine orange > acriflavine > brilliant cresyl blue.

T2, T4, and T6: all the dyes were inactive in the T-even phages.

The data seems to suggest that there is a specific configuration for the dye-receptor site (substance-X) of related phages. A dye to be active must possess a suitable complimentary structure. Phage strains belonging to the same serological group show the same sequence of photodynamic activity for active dyes. These results are completely in agreement with Burnet's concept (1933) concerning the correlation of serological grouping and photodynamic susceptibility of phages.

Effect of dilution of active dye-phage mixture. The phage T5 was mixed with M/60,000 methylene blue in saline buffer diluent at pH 7.4, and after 20 min and 60 min incubation at room

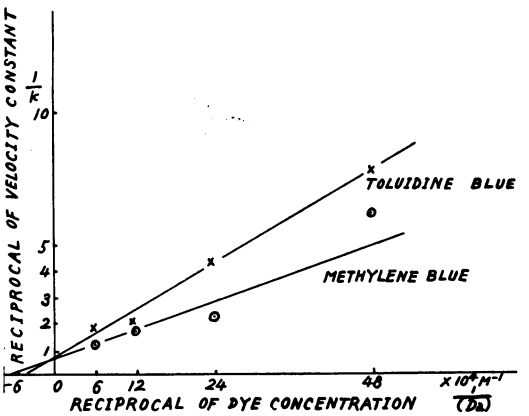

Figure 1. The effect of active dye concentrations on the photodynamic inactivation rate of coliphage T5 at pH 7.4.

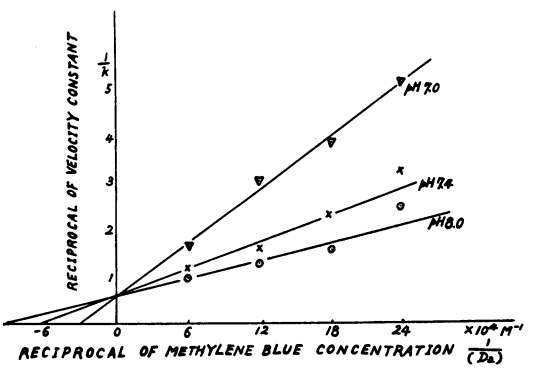

Figure 2. The effect of methylene blue concentration on the photodynamic inactivation rate of coliphage T5 at pH 7.0, pH 7.4, and pH 8.0.

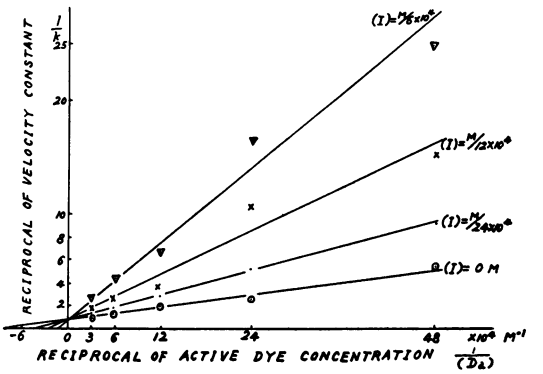

Figure 3. The kinetics of competitive inhibition on the photodynamic inactivation of coliphage T5. Active dye: methylene blue. Inhibiting dye: crystal violet. pH 7.4.

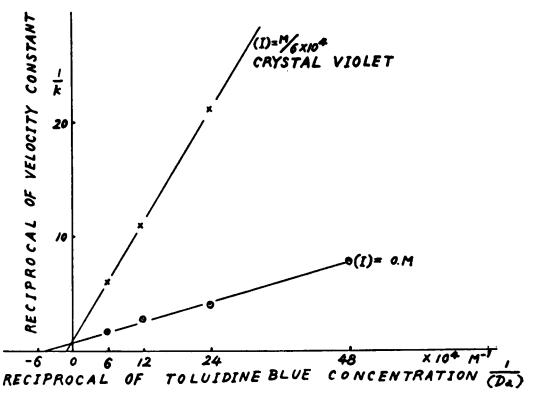

Figure 4. The kinetics of competitive inhibition on the photodynamic inactivation of coliphage T5. Active dye: toluidine blue. Inhibiting dye: crystal violet. pH: 7.4.

temperature, the mixture was diluted 10^{-1} and 10^{-2} in saline buffer. The PI of this diluted mixture was negligibly small. Thus, the combination of the dye with receptor site of the phage, if it occurs, must be readily reversible. Welsh and Adams (1954) obtained similar results for phage T1.

Effect of dye concentration and pH on PI of T5. The effect of the concentration (D_a) of active dyes on PI of phage T5 is shown in figure 1. The reciprocal of the velocity constant of PI ($1/k$) was found to be linearly proportional to the reciprocal of the active dye concentration ($1/(D_a)$). As shown by Welsh and Adams (1954) for phage T1, the rate of PI of T5 was increased as the pH increases (figure 2).

Competitive action by triphenylmethane dyes. If the inactive dyes associate with substance-X,

TABLE 3

*Inhibiting (competitive) ability of crystal violet and methyl green toward photodynamic action of several active dyes on coliphages T3, T5**

Active dye	Inhibiting dye	T3	T5
Methylene blue	Crystal violet	+	+
	Methyl green	−	−
Toluidine blue	Crystal violet		+
	Methyl green		+
Acridine orange	Crystal violet		+
	Methyl green	+	+
Acriflavine	Crystal violet		+
	Methyl green		+
Brilliant cresyl blue	Crystal violet		+
	Methyl green		+

* + and − indicate presence or absence of inhibition.

they should inhibit the PI of active dyes. It was found that crystal violet, an inactive dye, inhibits PI of active dyes (figures 3 and 4). These results can be explained on the basis of a competition of the inhibitor with the active dye for the substance-X of the phage. The experimental results from various active dye-inhibitor systems are summarized in table 3. Methyl green did not inhibit the photodynamic action of methylene blue. This is probably due to an extremely strong affinity of methylene blue for phage substance-X.

DISCUSSION

The PI of phage requires the combination of a suitable dye with the phage particles. This combination is reversibly dissociable. The process appears to be similar to elementary enzyme kinetics, as described by the Michaelis-Menten equation. Application of Michaelis-Menten kinetics to PI of phage can be made in the following manner.

(a) The total concentration of substance-X (X_t), which is the potentially susceptible site of photodynamic action on the phage, can be expressed as the sum of the concentration of those sites that are combined with active dye (XD_a) and those not combined (X).

$$(X_t) = (X) + (XD_a) \tag{1}$$

In the first approximation one may assume that the concentration of (X_t) is a constant fraction of the phage.

(b) If it is assumed that the velocity constant k of photodynamic action is proportional to the concentration of active dye-phage complex (XD_a) and reaches a maximum k_m when all susceptible sites of phages are so combined, then the initial reaction rate in a solution containing a known concentration of active dye and a fixed concentration of phage, may be regarded as being an estimate of k.

$$k \sim (XD_a) \qquad \frac{k}{k_m} = \frac{(XD_a)}{(X_t)} \tag{2}$$

(c) Assuming that the phage-dye coupling is a reversible association subject to the first order mass law equation,

$$\frac{(X)\cdot(D_a)}{(XD_a)} = K_a \tag{3}$$

where K_a = the dissociation constant of active dye-phage complex.

(d) Over a wide range of experimental conditions, the total concentration of the dye present is much greater than that of the susceptible sites on the phage or of the active dye-phage complex. The thermodynamic activity of the uncoupled active dye can be approximately represented by its total concentration (D_a).

(e) The presence of molecular oxygen is essential for the PI of phage. It is assumed that the concentration of molecular oxygen is sufficient for the oxidative step in the phage-dye photodynamic system.

(f) Using these five assumptions and substituting into the right-hand side of equation (2) the values indicated in (1) and (3), the following equation is obtained:

$$\frac{k_m}{k} = \frac{(X_t)}{(XD_a)} = 1 + \frac{K_a}{(D_a)} \tag{4}$$

This equation is linear for $1/k$ vs. $1/(D_a)$. The experiments of figures 1 and 2 indeed fulfill this requirement. The intercept of this line with the $1/(D_a)$ axis occurs at the point having the numerical value $-1/K_a$.

Expanding the theory to include the presence of varying amounts of a dissociable inhibitor competing for the sites of substance-X on the phage equation (4) must be extended to include the concentrations of the phage-inhibitor complex (XI).

$$(X_t) = (X) + (XD_a) + (XI) \tag{5}$$

Applying the mass action theory to the possible dissociations, one obtains:

$$\frac{(X)\cdot(D_a)}{(XD_a)} = K_a, \qquad (X) = (XD_a)\frac{K_a}{(D_a)}$$

$$\frac{(X)\cdot(I)}{(XI)} = K_I, \qquad (6)$$

$$(XI) = (X)\frac{(I)}{K_I} = (XD_a)\frac{(I)K_a}{(D_a)K_I}$$

where (I) = the concentration of inhibiting dye and K_I = the dissociation constant of phage-inhibitor complex. Substitution into the right-hand side of equation (5) the values indicated in (6), and applying the Michaelis-Menten concept of reaction velocity,

$$\frac{k_m}{k} = \frac{(X_t)}{(XD_a)} = 1 + \frac{K_a}{(D_a)} + \frac{(I)K_a}{(D_a)K_I} \qquad (7a)$$

$$= 1 + \frac{K_a}{(D_a)}\left\{1 + \frac{I}{K_I}\right\} \qquad (7b)$$

For $(I) = 0$, the equation reduces to (4). For a given value of (I), the equation predicts a linear relationship between $1/k$ and $1/(D_a)$. For a given value of (D_a) a linear relationship between $1/k$ and (I) is expected. As shown in figure 3, the experimental results fulfill these requirements. If one plots $1/k$ vs $1/(D_a)$ for various values of (I), a family of lines which meet at a point $1/(D_a) = 0$ and $1/k = 1/k_m$ should be obtained according to theory. In a $1/k$ vs (I) plot the lines corresponding to various values of (D_a) will meet at a point $(I) = -K_I$ and $1/k = 1/k_m$. The constants K_a, K_I, and k_m can thus be evaluated (Sumner and Myrbäck, 1950; Friedenwald and Maengwyn-Davies, 1954). The estimates obtained are given in table 4.

The following properties common to the active dyes (thiazine, oxazine, and acridine dyes) should be pointed out: (1) These active dyes are all basic. (2) These dyes are all hetero-tricyclic compounds. (3) These hetero-tricyclic compounds have two chargeable nitrogen atoms situated at comparable positions on both sides of the skeleton. (4) The distances of these two nitrogens are almost equal in all active dyes, and conjugated double bond systems lie between these two atoms (see table 1). (5) All of these active dyes possess an affinity for the nucleic acids and the association is of an ionic nature (Chargaff and Davidson, 1955).

These active dyes belonging to the three groups can all be derived from the fundamental skeleton by suitable variations in X and Y (X = N or C; Y = S, O, or N). The cations of these dyes are a resonance hybrid of the structures (figure 5, *Ia*, *Ib*, *Ic*). The cationic charge is located either on one of the two nitrogen atoms, or on the hetero-atom in the heterocyclic ring. This cationic charge associates with an anionic charge of the phage. The uncharged nitrogen atom in the side-chain forms a coordination bond with a cationic charge or an empty orbital via the lone pair of electrons (Mizushima, 1952; Dewar, 1948). Therefore,

TABLE 4

Dissociation constants of various dyes on coliphage T5

Dye and Action	pH	Dissociation Constant of Dye-Phage Complex
		M
Methylene blue (active dye)	7.0	3.23×10^{-5}
	7.4	1.35×10^{-5}
	8.0	1.03×10^{-5}
Toluidine blue (active dye)	7.4	1.96×10^{-5}
Acridine orange (active dye)	7.4	2.32×10^{-5}
Acriflavine (active dye)	7.4	1.85×10^{-5}
Brilliant cresyl blue (active dye)	7.4	1.89×10^{-5}
Crystal violet (inhibiting dye)	7.4	3.03×10^{-6}

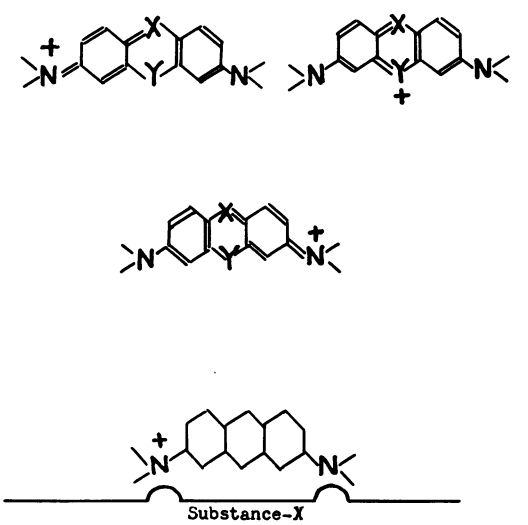

Figure 5. The common structures of active dyes. *Ia* (top left), *Ib* (top right), *Ic* (center): resonance. *II* (bottom): suggested mechanism of association with phage substance-X.

these active dyes may associate with the substance-*X* of phage through a bridge-like structure of the type shown (figure 5, *II*).

The following facts suggest that the substance-*X* of the phage may actually be its nucleic acid: (1) Both active dyes and inhibiting dyes have a strong affinity for nucleic acid (Chargaff and Davidson, 1955). (2) All of the T-even phages are very susceptible to osmotic shock as compared to the T-odd phages (Yamamoto, 1957). The pore-size of T-even phages is supposedly much smaller than that of T-odd phages. The experiments described indicate that the photodynamic susceptibility of phage strains is correlated with their permeability to dye molecules. Studies on the determination of substance-*X* as nucleic acid are now in progress.

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SUMMARY

The quinonimides such as the thiazine and oxazine dyes, and the acridine dyes were found to be very active in the photodynamic inactivation of the T-series of coliphages. The mechanism of inactivation appears to be a combination of active dye with phage through a bridge-like structure. Among the various inactive dyes tested, triphenylmethane dyes inhibit the photodynamic inactivation of phage by active dyes.

The combination of active dyes with the bacteriophages was found to be reversibly dissociable. Michaelis-Menten analysis satisfies the active dye-phage inactivation. Application of kinetics to the inhibition of the photodynamic action revealed that the inhibition is a competitive one analogous to that observed in the case of enzyme-inhibitor systems.

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